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Introduced or glacial relict? Phylogeography of the cryptogenic tunicate *Molgula manhattensis* (Ascidiacea, Pleurogona)

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ABSTRACT

Aim The tunicate *Molgula manhattensis* has a disjunct amphi-Atlantic distribution and a recent history of world-wide introductions. Its distribution could be the result of regional extinctions followed by post-glacial recolonization, or anthropogenic dispersal. To determine whether the North Atlantic distribution of *M. manhattensis* is natural or human-mediated, we analysed mtDNA cytochrome *c* oxidase subunit I (COI) sequence variation in individuals from cryptogenic and introduced ranges.

Location North Atlantic Europe and America; Black Sea; San Francisco Bay; Osaka Bay.

Methods Nuclear 18S rDNA sequences were used to resolve phylogenetic relationships and mtDNA COI sequences for phylogeographic analyses.

Results Phylogenetic analyses confirmed that *M. manhattensis* and *M. socialis*, which are frequently confused, are distinct species. MtDNA haplotype diversity was nearly three times higher with deeper relationships among haplotypes on the North-east American coast than in Europe. Diversity declined from south to north in America but not in Europe. In areas of known introductions (Black Sea, Japan, San Francisco Bay), *M. manhattensis* showed variable levels of haplotype diversity. Medium-to-high-frequency haplotypes originating from the North-west Atlantic were present in two locations of known introductions, but not in Europe. Private haplotypes were found on both sides of the Atlantic and in introduced populations. The mismatch distribution for the North-east Atlantic coast indicates a recent expansion.

Main conclusions *Molgula manhattensis* is native in North-east America. However, whether it was introduced or is native to Europe remains equivocal. Additional sampling might or might not reveal the presence of putative private European haplotypes in America. The low European diversity may be explained by low effective population size and a recent expansion, or by low propagule pressure of anthropogenic introduction. Absence of medium-to-high-frequency American haplotypes in Europe may be the result of exclusive transport from southern ports, or long-term residence. These arguments are ambiguous, and *M. manhattensis* remains cryptogenic in Europe.

Keywords

Amphi-Atlantic distribution, anthropogenic introduction, cryptogenic species, mt-COI, phylogeography, tunicate.

INTRODUCTION

Biological invasions are an important component of global change (Carlton, 2000; Harley *et al.*, 2006). They are a major threat to coastal marine biodiversity, community structure and ecosystem function (Chapin *et al.*, 2000; Bax *et al.*, 2003) because of their growing magnitude in conjunction with global trade and associated transport vectors (Carlton &

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Cohen, 2003), which are greatly accelerating the rate of non-indigenous species introductions into coastal communities (e.g. Ruiz *et al.*, 1997).

The impact of biological invasions on coastal communities increases with the rate of introductions; however, the notion that biological invasions are primarily a phenomenon of the 20th century has been tempered as a result of seminal work by Carlton (1979). Humans have been moving coastal species across the North Atlantic for a thousand years starting with the Viking explorations. From the 16th century onwards, opportunities for introductions in both directions increased dramatically with the onset of intensive shipping and emigration. These events occurred long before the first coastal surveys were carried out by naturalists in the mid-1800s (Carlton, 2003a). A well-studied example is the periwinkle, *Littorina littorea*, which was introduced to America from Europe probably in the 18th century with rock ballast (Blakeslee *et al.*, 2008; Brawley *et al.*, 2009).

Cryptogenic species

These unrecorded historical introductions contribute to today's cryptogenic species, i.e. species that are neither demonstrably native nor demonstrably introduced (Carlton, 1996). The total number of cryptogenic species is greatly underestimated, as we generally assume that species are native, unless they are proven to be introduced (Carlton, 2008). Studying the phylogeography of cryptogenic species can aid in estimating the impact of anthropogenic vectors on the dispersal and biogeography of coastal biota.

Phylogeography in the North Atlantic

The ranges of both terrestrial (Hewitt, 1996) and coastal (Maggs *et al.*, 2008) organisms across the North Atlantic have been significantly shaped by the last glacial maximum (LGM) ($\sim$ 20,000 years ago). Phylogeographic studies of marine organisms have revealed a general pattern of post-glacial expansion into northern regions from predominantly southern, peri-glacial refugia along both European and North American coasts (reviewed by Maggs *et al.*, 2008). Because the effects of the LGM were generally more severe along North-west Atlantic shores, many species became locally extinct (Wares & Cunningham, 2001; Vermeij, 2005; but see Olsen *et al.*, 2010). North-west Atlantic shores were only subsequently recolonized from either regional refugia, or from the North-east Atlantic at the end of the LGM, with mid-Atlantic islands such as Iceland and Greenland typically recolonized from Europe and acting as stepping stones for recolonization of the North-west Atlantic, resulting in amphi-Atlantic species distributions (Ingólfsson, 1992).

Species with a continuous amphi-Atlantic distribution occur on European and American coasts of the North Atlantic, including northern Norway, Iceland, southern Greenland and North-east Canada. A disjunct amphi-Atlantic distribution is characterized by an absence of the species in Arctic waters, while occurring on European and American coasts, and is dependent on the long distance dispersal (LDD) capacities of the organism. Natural LDD can occur through larval transport on ocean currents and/or rafting of egg-masses, juveniles and adults. If, however, life history traits preclude LDD (as is the case with many shallow-water taxa) and anthropogenic vectors have been at work (such as shipping and translocation of shellfish), then the disjunct distribution is probably human mediated.

Molgula manhattensis: a cryptogenic tunicate

The Ascidiacea (Pleurogona, Urochordata) are a class of tunicates commonly referred to as sea squirts. They have a nondescript, sac-like body and are filter feeders (Fig. 1a). Their natural dispersal potential is low. Of the 184 shallow-water species in the North Atlantic, 16 have a disjunct amphi-Atlantic distribution. Five extend their distribution to warm or deep waters, eight are known to have been introduced on either or both Atlantic coasts and three are cryptogenic. One of these is the sea grape, *Molgula manhattensis*, which was first described from New York harbour by De Kay in 1843. Its North-west Atlantic distribution extends from Cape Cod to southern Louisiana (interrupted by the Florida peninsula) (Van Name, 1945). Although its European distribution extends from Norway to Portugal, it is patchy (Monniot, 1969).

Ascidian taxonomists have inferred human-aided transport for both European and North American *M. manhattensis* populations because of its occurrence in fouling communities, its seasonally high local densities and its patchy distribution (Van Name, 1945; Monniot, 1969). Moreover, *M. manhattensis* has a recent history of world-wide introductions, having been reported from the Mediterranean (Monniot, 1969), Aegean (H. Aslan, pers. comm.), Adriatic and Black Seas (D. Micu, pers. comm.), from California to Washington State on the Pacific coast of North America (Lambert, 2001), Japan (Tokioka & Kado,1972), Vladivostok (Zvyagintsev *et al.*, 2003) and Australia (Kott, 1985). The inferred anthropogenic vectors for these introductions are hull fouling and oyster translocations (Tokioka & Kado, 1972; Cohen & Carlton, 1996), and possibly ballast water (Hewitt *et al.*, 2004).

Habitat and dispersal potential

Molgula manhattensis occurs on hard substrates, ranging from shells in an otherwise muddy environment, to rocky shores. It commonly occurs on American oysters (*Crassostrea virginica)*, and it can seasonally be the most abundant oyster-fouling organism (Galtsoff, 1964). It is tolerant of high turbidity, organic content and polluted waters. It is reported from pontoons, dikes, buoys and ship hulls (Woods Hole Oceanographic Institution, 1952).

Ascidians are simultaneous hermaphrodites; sperm is released in the water column and eggs are fertilized internally. Self-fertilization, which is an advantage in LDD, occurs in

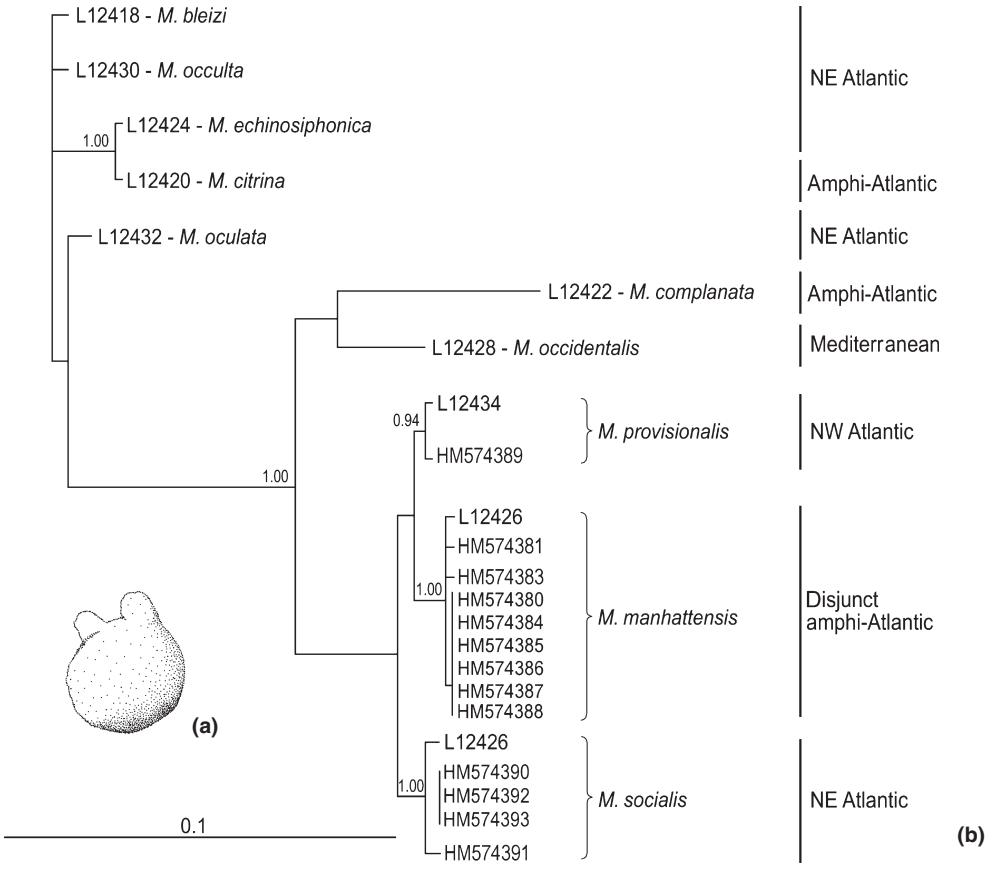

Figure 1 (a) *Molgula manhattensis,* size *c.* 1.5 cm. (b) 18S DNA Bayesian tree of *Molgula* spp. Species names and GenBank accession numbers are given for each sequence. Numbers on branches are Bayesian posterior probabilities (if ≥90%). The tree is rooted with *Molgula citrina, M. echinosiphonica, M. occulta and M. bleizi*. The scale bar represents the number of expected changes per site. For each species or species group, the generalized distribution in the North Atlantic Ocean is indicated.

several ascidian species including *M. manhattensis*, for which artificial self-fertilization has been documented in the laboratory (Morgan, 1942). There is no information on the incidence and frequency of self-fertilization in the field. Larval duration in ascidians is short, ranging from minutes to several hours (Svane & Young, 1989). *Molgula manhattensis* has tadpole larvae that can actively swim, whereas other molgulids have tailless, non-swimming larvae (Huber *et al.*, 2000). Rafting of eggs, juveniles or adults has not been reported (Thiel & Gutow, 2005). These life history traits make natural LDD unlikely, whereas LDD via hull fouling and with oyster translocations has been demonstrated (see above).

Here we ask whether the disjunct amphi-Atlantic distribution of *M. manhattensis* is natural or the result of anthropogenic introduction. Our null hypothesis is that *M. manhattensis* is introduced on the North-east Atlantic coast. To test this hypothesis, we (1) establish the identification and monophyly of *M. manhattensis* with its sister species; and (2) reconstruct its phylogeographic and demographic history by comparing individuals from both coasts of the North Atlantic, as well as samples collected from known introductions in other parts of the world.

METHODS

Sampling

Molgula manhattensis was sampled from 12 locations across its range including putatively natural and non-natural locations (Table 1). Samples were collected from ropes and floating docks. Individuals were collected at least a few metres apart and placed immediately in 95% ethanol. A piece of the gonadal tissue was used for DNA extraction.

In Europe, *M. manhattensis* is often confused with the morphologically similar and closely related *Molgula socialis* (Arenas *et al.*, 2006). Both occur in the same habitat, although *M. manhattensis* seems to be more euryhaline. To compare the levels of intra- and inter-specific genetic diversity, two populations of *M. socialis* (Table 1) and a single individual of *Molgula provisionalis* from Hudson Bay, Canada, were included in this study.

To further verify the taxonomic identification of 19th century collection records from both sides of the Atlantic, the DNA of a formalin-preserved specimen of *M. manhattensis* (National Museum of Natural History Naturalis, the Netherlands;

Table 1 *Molgula manhattensis* and *Molgula socialis* sampling locations.

Region and location	Latitude–longitude
Molgula manhattensis	
Europe	
Sylt, Germany	55°01′ N–8°43′ E
Delfzijl, the Netherlands	53°32′ N–6°92′ E
Grevelingen, the Netherlands	51°74′ N–3°89′ E
Oostende, Belgium	51°22′ N–2°94′ E
Le Havre, France	49°48′ N–0°12′ E
North America	
Woods Hole, Massachusetts	41°55′ N–70°54′ W
Mystic River, Connecticut	41°35′ N–71°96′ W
Long Island Sound, Bridgeport, Connecticut	41°17′ N–73°18′ W
Chesapeake Bay, James River, Virginia	36°94′ N–76°33′ W
Introduced range	
Black Sea, Bulgaria	44°16′ N–28°64′ E
Osaka Bay, Japan	34°68′ N–135°38′ E
San Francisco Bay, California	37°85′ N–122°48′ W
Molgula socialis	
Europe	
Oosterschelde, the Netherlands	51°67′ N–3°75′ E
Oléron Island, France	46°03′ N–1°37′ W

Invertebrate Collection, accession number 336, under *Molgula macrosiphonica*) collected in 1878 from the former Zuiderzee (now Lake IJssel) in the Netherlands was also extracted.

DNA extraction, amplification and sequencing

DNA extraction was performed with a CTAB protocol according to Hoarau *et al.* (2002).

Nuclear 18S rDNA gene

To confirm species identities and clarify inter-specific relationships, we sequenced a 1-kb section of the 18S rDNA gene for four *M. socialis*, nine *M. manhattensis* and one *M. provisionalis*, selected to represent different mt-cytochrome *c* oxidase subunit I (mt-COI) haplotypes (see below) and locations. The fragment was amplified using the primers 18SA 5′-AGCAGCCGCGGTAATTCCAGCTC-3′ and 18SB 5′-AAAGGGCAGGGACGTAATCAACG-3′ (Wada *et al.*, 1992). For the phylogenetic analysis, all PCR reactions consisted of 25-μL reaction volumes containing 2–20 ng DNA, 1× reaction buffer (5PRIME), 0.2 mM of each dNTP, 0.5 U HotMaster Taq DNA polymerase (5PRIME), and 0.5 μM of each primer. PCR was performed in a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA). The reaction profile was 2 min at 94 °C followed by 40 cycles of: 20 s at 94 °C, 20 s at 66 °C, 2 min at 65 °C; and a final elongation step at 65 °C for 10 min. PCR products were cleaned using ExoSapIt (USB Corporation) enzyme following the provider's instructions. Both strands were cycle-sequenced using the dGTP Big Dye Terminator kit (Applied Biosystems), purified on a Sephadex G-50 fine Column (Sigma-Aldrich) and run on an ABI 3730 gene analyzer.

Mitochondrial COI gene

For the phylogeographic analyses, we compared variation in the mitochondrial cytochrome *c* oxidase subunit I (COI) subregion for 244 *M. manhattensis* and 41 *M. socialis*. The COI subregion is highly polymorphic in most ascidian species investigated so far and has been a successful tool in the identification of previously unrecognized or cryptic ascidian invasions (e.g. Lopez-Legentil *et al.*, 2006). The universal primers HCO2198 and LCO1490 (Folmer *et al.*, 1994) were initially used to amplify a segment of the mitochondrial COI gene, and based on these sequences specific primers were developed for *M. manhattensis:* MMCO1F 5′-TCCGCTT TGAGTGGAGTTTT-3′ and MMCO1R 5′-AGATTGGATC TCCCCCTCCT-3′, and *M. socialis:* MSCO1F 5′-TGGTACGA TAGCAGCGCTTA-3′ and MSCO1R 5′-TAGGATCTCTCCC TCCAGCA-3′.

All PCR reactions consisted of 50-μL reaction volumes containing 2–20 ng DNA, 1× reaction buffer (Promega, Madison, WI, USA), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.25 U Taq DNA polymerase (Promega), 0.15 μM of each primer and 0.1 mg mL^{-1} Bovine Serum Albumin (BSA). PCR was performed in either a Gene-Amp-System 9700 (Perkin-Elmer, Waltham, MA, USA) or a MyCycler (BioRad, Hercules, CA, USA). The reaction profile was 2 min at 94 °C followed by 40 cycles of: 20 s at 94 °C, 30 s at 52 °C and 90 s at 72 °C; and a final elongation step at 72 °C for 7 min. PCR products were cleaned, and both strands were cycle-sequenced as for 18S.

Data analyses

18S rDNA sequences were aligned using BioEdit v.7.0.5 (Hall, 1999) with a final alignment length of 965 bp. Sequences were analysed with MrBayes v 3.1 (Ronquist & Huelsenbeck, 2003). The optimal model of sequence evolution for the Bayesian analysis was determined using ModelTest v 3.7 (Posada & Crandall, 1998). The following parameters were used in the Bayesian analysis: model of sequence evolution = GTR + Γ, generations = 3,000,000, burn-in = 1,000,000. Trees were rooted using published sequences of *Molgula bleizi*, *M. citrina*, *M. complanata* and *M. echinosiphonica*. *Molgula occidentalis*, *M. oculata* and *M. occulta* sequences were also included for reference (Hadfield *et al.*, 1995).

CO1 haplotype sequences were aligned in BioEdit v.7.0.5 (Hall, 1999); there were no gaps in the sequences. The final fragment length was 550 bp for *M. manhattensis* and 583 bp for *M. socialis*, the extra 33 bp for *M. socialis* occurred at both the 3′ and 5′ ends of the sequence.

Estimates of haplotype (*h*) and nucleotide diversities (π) were performed with DnaSP v 4.10.9 (Rozas & Rozas, 1995).To compare haplotype diversities across sampling locations, rarefaction was used to correct for unequal sample sizes

($n = 20$) using HPRare v 1.0 (Kalinowski, 2005). Statistical testing was performed with the software Fstat v 2.9.3.2 (Goudet, 1995). Haplotype richness estimates were performed using Estimates v 8.0.0 (Colwell, 2006). Estimates calculates a nonparametric estimator, Chao2, that can be used to predict the eventual asymptote in haplotype diversity for a certain number of samples in a region. The Chao2 estimator includes the effects of private or rare haplotypes on the total haplotype diversity. The greater the number of rare haplotypes, the more likely it is that haplotypes that are in fact present, were not sampled (Gotelli & Colwell, 2001). Estimated haplotype richness (Chao 2) was plotted against the number of samples for pooled North-east American and European populations.

Haplotype networks were inferred using statistical parsimony in TCS v. 1.13 (Clement *et al.*, 2000).

Mismatch distributions, time since divergence (t and Tau) and tests for population growth and expansion (Theta pi, Tajima's D and Fu's Fs) were estimated in Arlequin v 3.5 (Excoffier *et al.*, 2005). Mismatch distributions use tree shape to provide a rough estimate of population expansion or contraction as a result of a bottleneck. Tau is a measure of population age or time since expansion and is equal to $2(\mu)t$, where μ is the divergence rate and t is the generation time. Although these values are not known for any *Molgula* species, we estimated μ as [550 (sequence length) $\times$ 0.01 $\times$ 10 $-$ 6 (divergence rate of 1%/million yrs) $\times$ 2 (generation time)] = 0.000011. The actual values are not so important as the relative values are compared on both sides of the Atlantic. Θ_π (a function of $N_e$female), Tajima's D and Fu's Fs provide additional trends with respect to equilibrium and non-equilibrium conditions and population expansion. Negative values indicate putative population expansion; positive values putative contraction.

RESULTS

Phylogeny

Phylogenetic analysis of 23 18S sequences resolved the three species of interest (*M. provisionalis*, *M. manhattensis* and *M. socialis*). There was no evidence of misidentifications or cryptic species (Fig. 1). GenBank accession numbers are given in Fig. 1.

Haplotype diversity and private haplotypes

Among 550 base pairs, 34 were polymorphic. Nucleotide diversities were an order of magnitude higher in the southern populations on the Atlantic coast of North America and in San Francisco Bay when compared with all Europe. All mutations except one (in an individual from Long Island Sound) were at the third codon position.

The mtDNA diversity was moderately high with 31 haplotypes recovered from the 244 *M. manhattensis* individuals (Table 2, Fig. 2). The GenBank accession numbers for haplotypes 1–31 are HM574345 to HM574375. In Europe, the total

Table 2 Sampling locations, haplotypes per site and diversity measures for samples of *Molgula manhattensis* and *Molgula socialis*.

Region and location	N	Haplotype codes	n_h	n_{hc}	n_p	n_{pc}	h	N_p	π
Molgula manhattensis									
Europe									
Sylt	21	H1, H18	2	2.00	1	1.00	0.095	2	0.00035
Delfzijl	22	H1, H6, H16	3	2.99	2	1.99	0.385	3	0.00090
Grevelingen	21	H1	1	1.00	0	0.00	0.000	0	0.00000
Oostende	21	H1, H3, H14, H15	4	4.00	2	2.00	0.471	3	0.00094
Le Havre	20	H1, H3, H17	3	3.00	1	1.00	0.279	2	0.00053
North America									
Woods Hole	22	H2, H12	2	1.99	0	0.02	0.091	1	0.00017
Mystic River	21	H1, H2, H5, H31	4	4.00	2	2.00	0.586	7	0.00483
Long Island Sound	24	H1, H2, H3, H10, H11, H12, H30	7	6.93	2	1.99	0.768	6	0.00238
Chesapeake Bay	28	H1, H3, H4, H8, H9, H21, H22, H23, H24, H25, H26, H27, H28, H29	14	13.21	11	10.29	0.817	15	0.00266
Introduced range									
Black Sea	6	H1, H19	2	–	1	–	0.333	1	0.00061
Japan	16	H1, H4	2	–	0	–	0.125	1	0.00023
San Francisco Bay	22	H1, H3, H4, H7, H10, H13, H20	7	6.99	3	3.02	0.840	6	0.00290
Molgula socialis									
Europe									
Oosterschelde	21	HA, HB	2	–	0	–	0.181	3	0.00093
Oléron Island	20	HA, HB, HC	3	–	1	–	0.195	4	0.00069

N, number of individuals per location; n_h, number of haplotypes; n_{hc}, number of haplotypes after rarefaction to 20 individuals; n_p, number of private haplotypes; n_{pc}, number of private haplotypes after rarefaction to 20 individuals; h, haplotype diversity; N_p, number of polymorphic sites; π, nucleotide diversity.

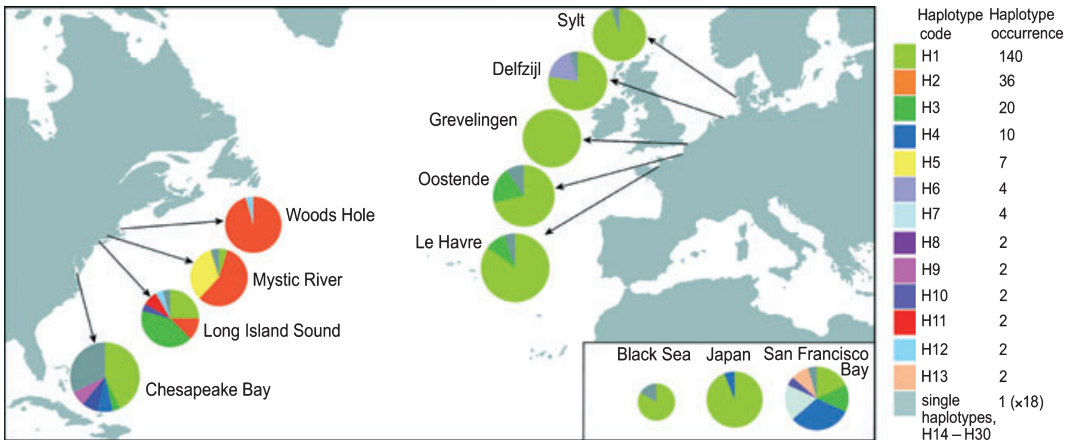

Figure 2 Distribution of CO1 haplotypes per sampling location of *Molgula manhattensis*. Colours correspond to haplotypes (see legend and Table 2). Grey-shaded areas are the cumulative proportion of single, unique haplotypes per location. The legend gives occurrence of haplotypes across all locations.

number of haplotypes was 8 (corrected haplotype richness = 7); the total number of haplotypes in America was 21 (corrected haplotype richness = 20). This included the two shared haplotypes H1 and H3 (Table 2).

Haplotype richness (n_h) was higher than the average (average n_h = 4.25; corrected average n_{hc} = 4.61) for populations in Long Island Sound, Chesapeake Bay and San Francisco Bay; all other locations had a lower than average number of haplotypes (Table 2). Haplotype diversity decreased from south to north along the Atlantic coast of North America; in Europe, no such pattern was found. Of the three sampled populations that are known introductions, two exhibited low haplotype richness and one (San Francisco Bay) had high haplotype richness (Table 2).

Private haplotypes were present in all regions, with corrected numbers of 16 in North America, six in Europe and four in the introduced range. The North American and European numbers did not differ significantly (Mann–Whitney *U*-test).

Diversity against sampling effort was compared for both sides of the Atlantic (Fig. 3). In Europe, the mean expected haplotype richness for five sampled populations was 20, compared to the eight observed. In North-east America, 93 haplotypes were predicted for four sampled populations compared to the 21 observed. The sampling effort did not capture the actual diversity – especially on the American side. Nevertheless, the observed haplotype richness in America was still 2.6 times greater than in Europe, and the expected haplotype richness was 4.6 times greater. This relationship would not be expected to change with more sampling.

The COI sequence of the formalin-preserved *M. manhattensis* museum specimen from the Netherlands confirmed that it was indeed *M. manhattensis*. Because of the degradation of the DNA, the sequence was not used in phylogeographic analyses. The COI sequence of the single *M. provisionalis* individual from Hudson Bay (HM574376) corresponded to haplotype H1, but the 18S data indicate that *M. provisionalis* and *M. manhattensis* are not the same species (Fig. 1).

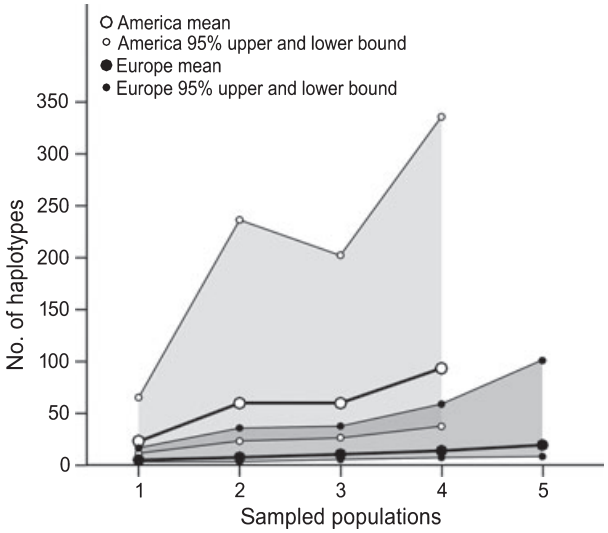

Figure 3 Haplotype estimation curves for European and North-east American populations of *Molgula manhattensis* using ESTIMATES (Colwell, 2006). The European Chao2 estimator suggests a maximum expected number of haplotypes of 20 (95% Confidence interval: 9–100), the North-east American maximum expected number of haplotypes is 93 (95% Confidence interval: 38–334).

Haplotype networks

The central haplotype (H1) in Fig. 4(a) accounts for 55% of *M. manhattensis* individuals sampled, 86% of those from Europe and 20% of the North-east American individuals. H1 was present at all locations except Woods Hole. All European haplotypes were within one or two point mutations from the central haplotype, whereas the North-east American haplotypes ranged from one to five steps from H1. The relationships of North-east American haplotypes are, therefore, deeper and older. High-frequency nested North-east American haplotypes were present in the introduced range, but were absent in European populations.

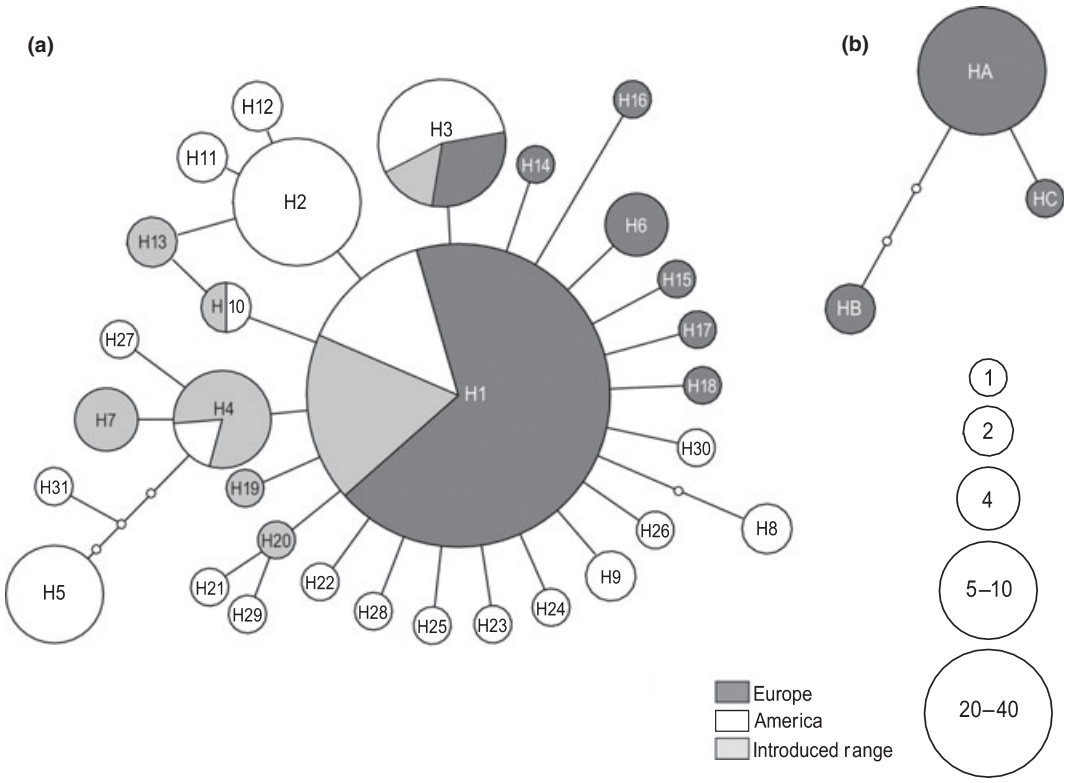

Figure 4 Haplotype networks for *Molgula manhattensis* (a) and *Molgula socialis* (b). Numbers represent haplotype identities (see Table 2). Haplotype circles are proportional to haplotype frequency, see legend. European haplotypes (or proportions of haplotype occurrence) are indicated in black, North-east American haplotypes are white and haplotypes in the introduced range are grey.

For *M. socialis* (Fig. 4b), the 42 sequences yielded only three haplotypes (HA-HC; GenBank accession numbers HM574377–HM574379) and low diversity (Table 2). The depth of the haplotype network was similar to that of the European *M. manhattensis*; the maximum distance from the central haplotype (HA) was three mutations (Fig. 4b).

Historical demography and divergence

The distribution of the frequencies of observed numbers of differences between pairs of haplotypes for each region is shown in the mismatch distributions (Fig. 5). The North-west Atlantic populations exhibit a bimodal distribution consistent with population differentiation, two possible refugia and possibly admixture, characteristic of long native residence. Tajima's D and Fu's Fs further support expansion under non-equilibrium conditions (Table 3). The San Francisco Bay mismatch distribution is unimodal and represents a subset of the North-west Atlantic distribution including population expansion. However, Tajima's D and Fu's Fs are not significant. The North-east Atlantic mismatch distribution also indicates recent expansion as do Tajima's D and Fu's Fs, both of which are significant. Estimates of the time since expansion (Table 3) based on Tau and Theta Θ_π suggest a smaller female effective population size and an earlier expansion in Europe than America (and the introduced population in San Francisco Bay), although the confidence intervals overlap, reducing the reliability of the relative estimates.

DISCUSSION

The North Atlantic Ocean is the centre of molgulid diversity, with 31 described shallow-water species. Four of these are restricted to the North-west Atlantic and 14 to the North-east Atlantic. Ten occur in Arctic waters and two have amphi-Atlantic distributions. *M. manhattensis* is the only one with a disjunct amphi-Atlantic distribution.

Phylogeny

The 18S phylogeny (Fig. 1) confirms that our identifications were correct and that the currently recognized *Molgula* species form highly supported monophyletic groups (Hadfield *et al.*, 1995; Huber *et al.*, 2000). On the North-west Atlantic coast, *M. provisionalis,* which has only been recognized as a separate species since 1945, replaces *M. manhattensis* north of Cape Cod (Van Name, 1945). Its monophyly based on 18S data is confirmed. The single *M. provisionalis* for which we have a COI sequence shared the *M. manhattensis* ancestral haplotype H1, which could reflect past introgression. On the North-east Atlantic coast, *M. manhattensis* and its close relative *M. socialis* occur in the same area and habitat, sometimes even in the

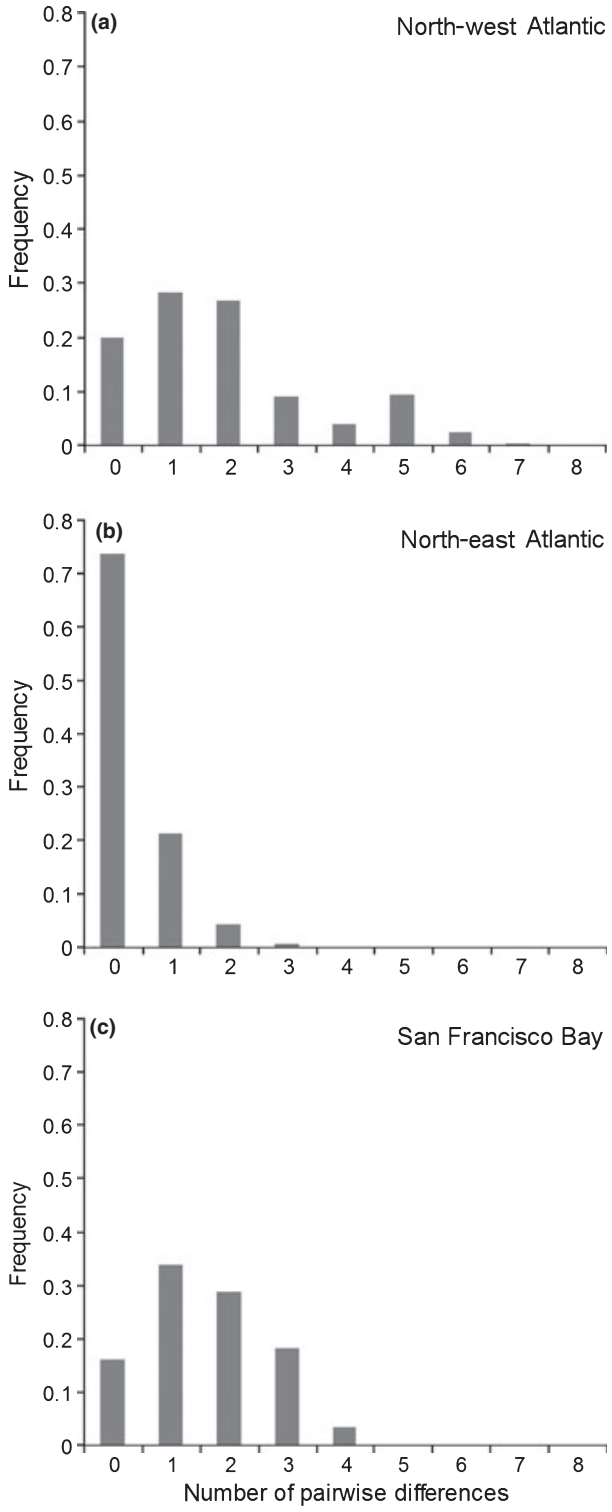

Figure 5 Comparative mismatch distributions for *Mogula manhattensis* in the (a) North-west Atlantic, (b) North-east Atlantic and (c) San Francisco Bay.

same location. *M. socialis* has frequently been misidentified as *M. manhattensis* (Arenas *et al.*, 2006), because species-specific anatomical characters can only be seen after detailed dissection (Monniot, 1969).

The closest relative of *M. manhattensis* appears to be *M. provisionalis*, followed by *M. socialis;* however, the posterior probability supporting the three clades is < 90% so that we cannot be certain that the sister-group relationships shown are correct. As a consequence, the biogeographic patterns of the species may or may not be correlated with the sister clades shown in Fig. 1. Therefore, based on the phylogeny, *M. manhattensis* could have occurred naturally on both sides of the Atlantic, precluding support or rejection of our null hypothesis of introduction to Europe.

Historical records

Baster (1762) described a *Molgula* species from the Netherlands, possibly the first record of *M. manhattensis* in Europe. The species was reported in both Europe and North-east America from the 19th century onward, as was confirmed by morphological comparison of 19th century specimens from both Atlantic coasts (Monniot, 1969). We have confirmed 19th century presence of *M. manhattensis* in Europe based on the COI sequence of the museum specimen collected in 1878 from the Netherlands. These records do not permit us to determine whether *M. manhattensis* is native or introduced in Europe.

Phylogeography and haplotype diversity in the North Atlantic

Comparison of haplotype richness and other diversity statistics across the Atlantic (Table 2) shows a consistently higher mean corrected diversity of nearly threefold on the American side when compared with Europe. Only the central ancestral haplotype (H1) and closely related H3 are found on both sides.

North-west Atlantic populations are strongly differentiated from one another (F_{ST} 0.328, $P < 0.001$) and show a latitudinal gradient of diversity from south to north (Fig. 2), consistent with post-LGM expansion from a southern refugium, possibly in the Chesapeake Bay region. At the same time, the dominant northern haplotype decreases in frequency southwards. This suggests a northern refugium, possibly in ice-free areas of Nova Scotia and Newfoundland, and a subsequent contact zone to the south in the Long Island Sound region. The mismatch distribution (Fig. 5) is consistent with admixture. As a hard substrate species, survival of *M. manhattensis* in southern refugia would have been difficult given the predominantly sandy coastlines (Wares & Cunningham, 2001; Wares, 2002; Maggs *et al.*, 2008). However, it may have taken advantage of growing on the shells of the American oyster, *Crassostrea virginica*, which has been present in North-west Atlantic waters since before the Pleistocene glaciations (Vermeij, 2005). In any case, the high diversity of the western Atlantic combined with nearly three times the number of haplotypes and, most significantly, the greater phylogeographic depth of American haplotypes, is consistent with North American native residency long before and after the LGM.

Table 3 Demographic parameters for *Mogula manhattensis*.

	North-west Atlantic	North-east Atlantic	San Francisco Bay
Tau	1.75 (0.07–2.89)	3.00 (0.39–3.50)	1.73 (0.93–2.73)
T since expansion (y)	79,367 (3285–139,300)	136,363 (16,700–159,090)	79,367 (42,270–124,030)
Θ_π	1.88	0.32	1.59
Tajima D	−1.8432**	−2.0230***	−0.0995 NS
Fu Fs	−12.7976***	−7.5230***	−1.7217 NS

See Methods for parameterization values. Negative values for Tajima's D and Fu's Fs indicate population expansion: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

North-east Atlantic populations of *M. manhattensis* are differentiated ($F_{ST} = 0.0716$, $P < 0.05$) but more weakly and do not show a latitudinal diversity gradient (Fig. 2). The central haplotype H1 accounts for 86% of the total number of sequences. This pattern is atypical for European rocky shores, where a number of high-diversity refugia have been identified, especially in north-west Iberia, Brittany and south-west Ireland (reviewed in Maggs *et al.*, 2008). There are no confirmed records of current populations of *M. manhattensis* in the British Isles or the Iberian Peninsula, and we were unable to find *M. manhattensis* at Oléron Island (Atlantic France), where all sampled individuals were *M. socialis.* Recently, *M. manhattensis* has been confirmed in Brittany (F. Viard, pers. comm.). We have no sampling in these more southerly locations, and whether higher diversity would be found is unknown. The weaker population differentiation indicates greater gene flow than among American populations. This could reflect relatively high recreationally mediated gene flow among marinas as for the invasive tunicate *Styela clava* in New Zealand (Goldstien *et al.*, 2010).

Patterns of haplotype diversity in introduced populations

Low genetic diversity because of a population bottleneck was originally thought to be a characteristic of introduced populations (Geller *et al.*, 2010). However, recent studies have shown that diversity of introduced populations can be similar to that of native populations and may even exceed native diversity as a result of admixture or high propagule pressure, i.e. a combination of the absolute number of individuals released in one introduction event and the number of release events (Simberloff, 2009). *M. manhattensis,* in its introduced range, shows both patterns.

The high level of haplotype diversity in San Francisco Bay is similar to that in Chesapeake Bay and Long Island Sound (Fig. 2, Table 2) and given that four of the seven haplotypes are unique to these two locations, it is quite certain that the San Francisco Bay introductions came from these sources. Thousands of ships with rich fouling communities sailed from the East to the West coast of the USA. Furthermore, between 1869 and 1940, large quantities of live oysters were transported by the transcontinental railway from New York and Long Island Sound to San Francisco, where they were placed in local waters for storage or growth and maturation. The ships and oyster translocations were also responsible for introductions of other fauna (Carlton, 1979; Miller, 2000).

In the case of *M. manhattensis*, sustained, high propagule pressure from oyster transplants to San Francisco Bay surely explains the high observed diversity. In contrast, diversity was low in Japan and the Black Sea, and the ancestral haplotype H1 was dominant. The origin of *M. manhattensis* in Japan was hypothesized to have been Atlantic Europe (Tokioka & Kado, 1972), but this is not supported by our data. The haplotypes present in Japan include H1, but also H4, which occurs in Chesapeake Bay and San Francisco Bay, and not in Europe. It is, therefore, more likely that *M. manhattensis* was introduced to Japan from the Pacific coast of the USA and indirectly from the US Atlantic coast. The origin of *M. manhattensis* in the Black Sea is Atlantic Europe as five of the six individuals were H1 and one individual was H19, a derived, local haplotype. The vector of introduction in these populations is probably hull fouling, which is reflected by the low haplotype diversities.

Private haplotypes

Geographically restricted or private haplotypes are an indicator of longer-term residency far exceeding the timeframe of human introductions (Wares, 2002). Private haplotypes were found in both North America and Atlantic Europe, consistent with long-term residence. However, we also found four putatively private alleles in introduced populations.

Our sampling did not completely capture the diversity estimated to be present – mainly in North-east America (Fig. 3); low-frequency North-west Atlantic haplotypes were missed. Further intensive sampling could reveal that the putatively private haplotypes in the introduced populations also occur in the source populations of the North-west Atlantic and thus did not evolve *in situ*. Likewise, if the European populations are the result of an introduction, then their putative private haplotypes could also be artefacts. However, the main argument against introduction to Europe is that we would also have expected to see the medium- and high-frequency Atlantic American haplotypes (e.g. H2 and H4, Fig. 4a) in Europe (given the number of locations sampled there), and we do not.

Historical demography and introduced populations

Estimates of expansion and divergence times are particularly sensitive to effective population size and its effects on genetic drift. We find evidence for expansion in both Europe and San Francisco Bay in the mismatch distributions (Fig. 5); but no evidence for expansion in San Francisco Bay based on Tajima's D or Fu's Fs, while there is evidence for non-equilibrium expansion in Atlantic America and Europe (Table 3). In general, if repeated or massive introductions have occurred, these calculations can give the impression of a relatively long presence and expansion that is not actually the case, e.g. in San Francisco Bay. In Europe, however, the expansion shape is also recent (possibly since the LGM) but Tau suggests a deeper divergence from possibly a low number of older surviving haplotypes.

Introduced or glacial relict?

Distinguishing between an anthropogenic introduction and post-glacial recolonization requires multiple lines of evidence. Life history traits, preferred habitat and a patchy distribution argue for an introduction of *M. manhattensis* from America to Europe. *Molgula manhattensis* has a low natural dispersal potential, and it is not clear how it would have spread naturally from America to Europe. The vector of introduction in Europe may have been hull fouling, in particular inside empty shipworm galleries or in bilges (J.T. Carlton, pers. comm.), as the first records of *M. manhattensis* in Europe pre-date the first American oyster transfers to Europe in the 1870s (Carlton & Mann, 1996). Moreover, although common in oyster culture areas in Belgium and the Netherlands, where it grows on docks, piles and other man-made structures, *M. manhattensis* is not found on the shells of the oysters *Ostrea edulis* (Korringa, 1951) and *Crassostrea gigas (*D. Haydar, unpublished data), as would be expected given that it commonly occurs on American oysters. Finally, for most coastal taxa, Europe is today more diverse in the number of species than North America (Briggs, 1995) mainly because post-glacial recolonization of the North American coast occurred from Europe via Iceland (Wares & Cunningham, 2001; Vermeij, 2005). Recolonization of Europe from North America has not been documented, nor are there examples of species that have a naturally disjunct amphi-Atlantic distribution. Together, these arguments support an anthropogenic origin of *M. manhattensis* in Europe.

The genetic data, however, present some challenges. Low haplotype diversity could be natural if the range and refugia on the European side were small. Comparison of the haplotype networks of *M. manhattensis* and *M. socialis* indicates a similar evolutionary history and low diversity (Table 2, Fig. 4). However, the number of *M. socialis* populations sampled was low, making this comparison weak. More importantly, the absence of haplotypes H2 and H4 in Europe is puzzling (Fig. 4a). If *M. manhattensis* was introduced in Europe, these should have been present, even with undersampling in Europe (Fig. 3). In fact, they were found in introduced populations where only single sampling took place. If *M. manhattensis* was introduced to Europe, the only explanation for the absence of H2 from the European side is exclusive anthropogenic transport from southern American ports, where H2 is less common. Finally, the presence of private haplotypes is not necessarily because of the undersampling, and the presence of a 2-step haplotype (H16) in Europe is supportive of native residence in Europe.

Final resolution of the question rests on relative strength of three inter-related factors: (1) the degree to which the total haplotypic diversity was sampled; (2) the effective population size of natural populations in refugia; and (3) the relative role of propagule pressure at a given location in space and time. First, more intensive sampling of the North-west Atlantic would certainly reveal more low-frequency haplotypes, which could include the putative private haplotypes found in Europe. More intensive sampling in Europe might reveal additional private haplotypes and possibly the missing medium-to-high-frequency American haplotypes; though, the latter is much less likely. Second, population size greatly affects our ability to distinguish invasions, bottlenecks and expansions. In particular, the recent expansion in Europe (Fig. 5) can still be consistent with long-term residence in Europe. This leaves us with propagule pressure and its effects. Given hull transport and relatively low propagule pressure over many decades in Europe, the haplotype diversity of European populations of *M. manhattensis* could remain low. In the most extreme case, a single introduction could have been successful involving, H1 and H3, and the 'private' European haplotypes. In contrast, the extremely high propagule pressure (involving tons of tunicate-carrying oysters) that occurred in San Francisco Bay over decades, probably accounts for the high diversity found there.

In conclusion, although one could consider more intensive sampling of the North-west Atlantic in combination with genotyping a nuclear marker (which would allow a test of linkage disequilibrium), it remains possible that there is no one answer. Observed patterns in Europe may be attributed to the natural post-LGM recolonization involving small populations and/or a low propagule-pressure introduction, one superimposed on the other. The arguments are ambiguous and fairly evenly weighted; *M. manhattensis* remains cryptogenic.

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BIOSKETCH

Members of the MarBEE group have a long-standing interest in the population genetics (J.L.O., W.T.S., G.H.), phylogeography (J.L.O., W.T.S., G.H.) and invasive species biology (D.H., W.J.W., J.L.O.). This research was part of D.H.'s PhD thesis in invasive marine species biology under the supervision of W.J.W.

Author contributions: D.H. conceived the ideas; D.H. and G.H. collected the data; D.H. and G.H. analysed the data; and D.H. and J.L.O. led the writing.

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